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Developmental Morphology of Irregularly-shaped Gametophytes of the Liverwort *Mizutania riccardioides* (Mizutaniaceae)

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Mizutania riccardioides Furuki & Z. Iwatsuki is unique in having an irregularly-branched, unistratose plant body (thallus). Although the resemblance between thalli of *Mizutania* and some fern gametophytes has been noted, the mode of development of the irregularly branching thalli of *Mizutania* has not yet been clarified. We examined the developmental morphology of thallus branching, focusing on the behavior of the apical meristem, which usually has a single apical cell that functions as an initial cell. When branched, the branch apical cells arise in the young merophytes derived from the original apical cell. This mode of branch apical cell formation is not unique and is found in other liverworts, but in *M. riccardioides* some branch apical cells stop dividing and disappear through periclinal divisions soon after initiation, while others give rise normally to branch thalli. Which branch apical cells are lost and which are retained as initial cells appears to be arbitrary. Furthermore, the apical cell in the growing branch thallus sometimes abruptly ceases to function and forms small branch thalli with fully differentiated non-meristematic cells. In conclusion, the arbitrary loss of apical cell activity and differences in duration and/or onset of the timing of branch apical cell activity results in highly irregularly shaped thalli.

Keywords: apical cell, apical meristem, branching, gametophytes, liverworts, *Mizutania riccardioides*

The monotypic liverwort family Mizutaniaceae Furuki et Z. Iwatsuki, which is found in the Malay Peninsula and Borneo, is unique in having irregularly branched, thalloid plant bodies (Furuki & Iwatsuki 1989). The thallus is unistratose throughout, with rhizoids arising from marginal cells. The thalli of *Mizutania* are similar to fern prothalli (gametophytes), especially to those of the Hymenophyllaceae and Vittariaceae (Crandall-Stotler *et al.* 1994), although thalli of *Mizutania* can be distinguished from fern thalli microscopically as they contain distinct brownish oil bodies and minute granules, and have a strongly and irregularly striolate verrucose surface (Furuki & Iwatsuki 1989). The systematic position of *Mizutania* has been ambiguous until recently, because no sporophytes had been recorded, but the presence of perichaetia surrounding the archegonia implies that the genus is a liverwort (Furuki & Iwatsuki 1989). Furthermore, the chromosome number (n = 9) and the karyotype suggest that *Mizutania* is closely related to the Metzgeriales (liverworts; Inoue & Furuki 1992), and recent molecular phylogenetic analyses using the *rbcL* gene have indicated that *Mizutania* belongs to the Jungermanniales, rather than to the Metzgeriales

(Masuzaki *et al.* 2007).

Irregularly branched thalli may be advantageous for increasing photosynthetic efficiency by preventing overlapping plant bodies. There may also be some developmental reason for the formation of irregularly shaped thalli. However, we still do not know the manner in which the thalli branch irregularly. Our aim was to clarify the branching mode of the thallus of *Mizutania* and to compare it to other bryophytes and fern gametophytes.

Materials and Methods

Thalli of *Mizutania riccardioides* Furuki et Z. Iwatsuki were collected in the Cameron Highlands, Malaysia, in November 2004, and fixed in FAA (stock formalin: glacial acetic acid:50% ethanol, 5:5:90) in the field. The thalli were dehydrated through a graded ethanol series, stained with safranin (0.8% in absolute ethanol) for 2 h, mounted in Permount (Fisher Scientific, NJ, USA), and observed under a light microscope (Olympus BX 51). The thalli are only one-cell thick, but because they are wavy, it can be difficult to obtain a sharp image; therefore, images taken at several focal points of a thallus were synthesized into a clear image using Photoshop CS.

In a well-branched thallus, branch thalli with a single apical meristem were regarded as individuals. For instance, the plant body in Fig. 1B comprises two individuals. The fully differentiated plant body in Fig. 1A was not regarded as an individual, because it had no thalli with an apical meristem. Young, regenerated buds growing from the thallus margin (Fig. 1A) were also not counted as individuals. In total, 186 individuals were distinguished in the present study. Voucher specimens (*T. Furuki & K.-T. Yong 19450*) are housed in the herbarium of the Natural History Museum and Institute, Chiba, Japan (CBM), and in the herbarium of the University of Malaya, Malaysia (KLU).

Results

Gross morphology of the plant body

Thalli of *Mizutania riccardioides* are narrow and elongated, with a highly irregular shape (Fig. 1A). Thallus branching generally occurs unequally (Fig. 1B–D), and the angles between the main and branch (lateral) thalli are not uniform (Fig. 1C, D). Thallus width varies greatly, even within a single thallus (Fig. 1A), suggesting differences in the growth ability of branch thalli meristems. Some branch thalli stop growing very early and have fully differentiated non-meristematic cells (Fig. 1C). As a result, it was often difficult to recognize which was the original branch among several branch thalli in a well-grown thallus (Fig. 1A).

In addition, regenerant buds (Furuki and Iwatsuki, 1989) arising from once-differentiated marginal cells contribute to the increasing irregularity of the thallus of *Mizutania* (Fig. 1A). Regenerant buds have a characteristic very narrow base (Fig. 1E) and easily drop from the thallus margins. Non-branched, and once- or twice-branched small thalli with acute bases, which are frequent in the field, are presumably derived from separated regenerant buds (Fig. 1B).

Some branched or unbranched large thalli with apical meristems (19 individuals) had female inflorescences (perichaetia) that arose from the margin rather close to the basal part of the thallus (Fig. 1C), suggesting that the thalli were mature. Gemmae arise from the margins of the fully differentiated basal part of the growing thalli, and also occur near the thallus apex if the thallus is well differentiated and has no apical meristem (Fig. 1C).

Development of apical branching

Young, unbranched, simple thalli, as well as growing branch (lateral) thalli, have single apical cells in their apical meristems. The apical cell

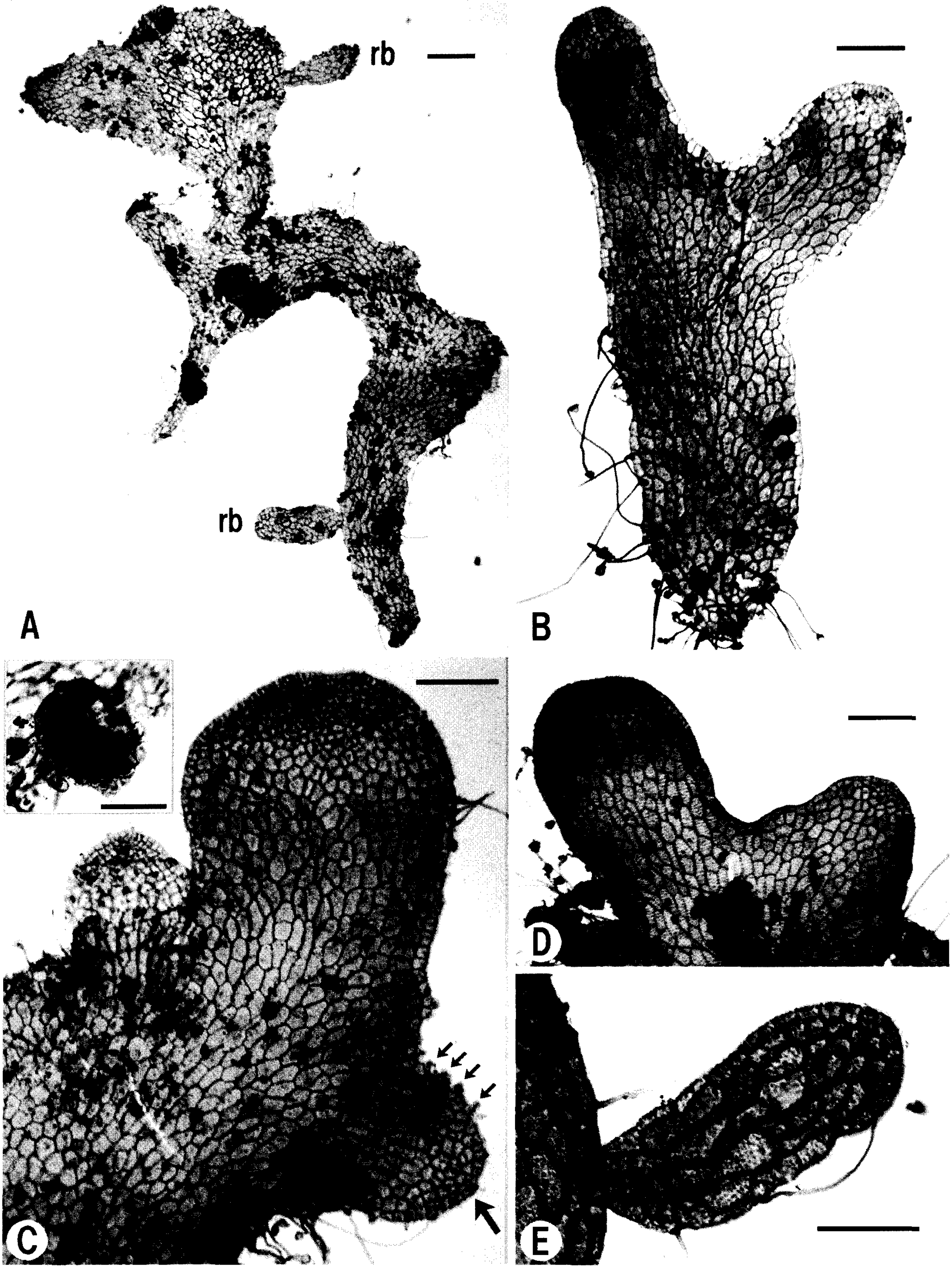

FIG. 1. Various thallus shapes of *Mizutania riccardioides*. A: Irregular thallus with regenerant buds (rb). B: Unequally branched, young thallus. C: Unequally branched thallus. Small arrows indicate gemmae arising from margin of fully differentiated thallus with no apical meristem (large arrow). Inset shows perichaetium arising from right basal portion of thallus. D: Unequally branched thallus. E: Regenerant bud arising from marginal cell. Scale bars = 200 μm for A–D, and 100 μm for inset of C and E.

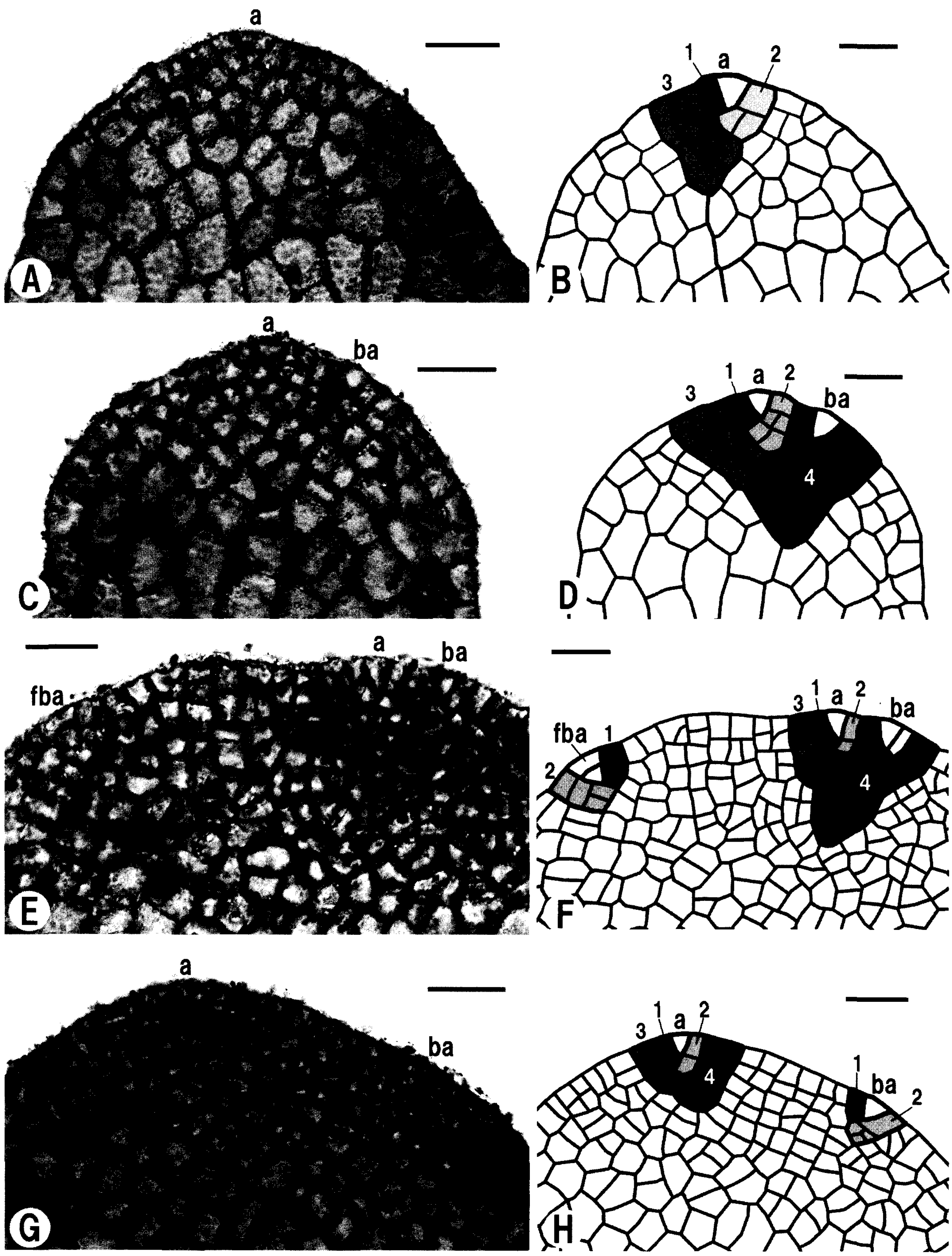

FIG. 2. Early stages of thallus branching in *Mizutania riccardioides*. Microscope images (A, C, E, G) and line drawings (B, D, F, H). First to fourth youngest merophytes are shaded in B, D, F, and H. A, B: Thallus tip with single apical cell. Enlarged image of apical portion of right thallus from Fig. 1D. C, D: Thallus tip with newly initiated branch apical cell. E, F: Thallus tip with developed branch apical cell with its own segment. Enlarged image of main thallus from Fig. 1C. Note that second youngest branch apical cell failed due to division in periclinal wall. G, H: Thallus with more developed branch apical cell. Enlarged image of left side of thallus of Fig. 1D. a, apical cell; ba, branch apical cell; fba, failed branch apical cell; 1–4, first to fourth youngest merophytes. Scale bars = 50 μm.

is triangular and cuts off segments from its two lateral faces (Fig. 2A, B). Each segment divides continuously in the periclinal and anticlinal planes to form a rectangular cell packet, generally called the merophyte. The merophytes were numbered from the youngest to oldest. In most cases, both

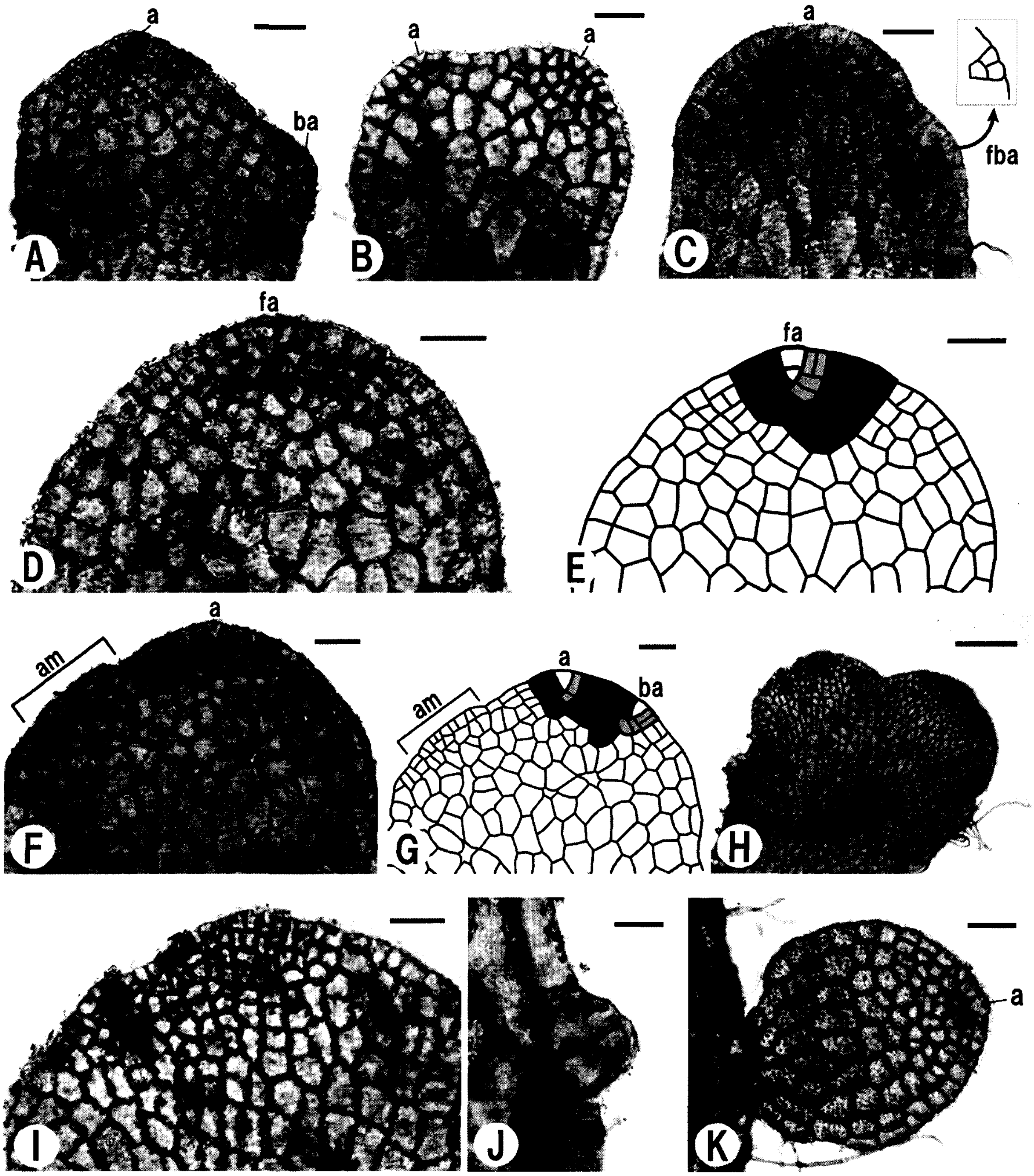

FIG. 3. Meristem behavior of *Mizutania riccardioides*. Later stages of thallus branching (A–C), thallus with failed apical cell (D, E), thallus with adventitious meristem (F–I), and regenerant bud (J, K). First to fourth youngest merophytes shaded in E and G. A: Unequally branched thallus. B: Equally branched thallus. C: Unequally branched thallus with failed apical meristem. Inset shows line drawing of failed apical cell. D, E: Thallus with failed apical cell with periclinal wall. E is line drawing of D. F, G: Thalli with adventitious meristems. G is line drawing of F. H, I: Nearly equally branched thallus. I: Enlarged image of left thallus in H to show adventitious meristem. J: Earliest stage of regenerant bud development. K: Young thalli derived from regenerant bud. a, apical cell; am, adventitious meristem; ba, branch apical cell; fa, failed apical cell; fba, failed branch apical cell. Scale bars = 50 μm for A–G, I, and K, 200 μm for H, and 20 μm for J.

the apical cell and adjacent young merophytes (first to fourth) are arranged so as to occupy a triangular area (e.g., Fig. 2B), suggesting that the apical segmentation pattern is very regular.

The apices of well-developed thalli form new apical cells in addition to the original apical cell (Fig. 2C, D). The new apical cell also cuts off segments from its two lateral faces (Fig. 2E, F), and concomitantly separates from the original apical cell, with intervening small cells between the two areas (Fig. 2G, H). Finally, the new apical cell gives rise to a new apical meristem of the lateral branch primordium (Fig. 3A). Therefore, the new apical cell can be regarded as a branch apical cell. In the present examination, just-initiated branch apical cells were always recognized in the fourth-youngest merophytes, suggesting that they are formed in sites somewhat apart from the original apical cell. If the growth of the branch and the original (main) apical cells occurs at a similar rate, the result is apparent equal (dichotomous) branching (Fig. 3B). Otherwise, unequal branching occurs (Fig. 1D).

Apical meristems with a single apical cell were found in 61% (113 of 186) of the individuals examined, and apical meristems with both original and branch apical cells were found in 33% (61 of 186) of all growing individuals. This latter type of meristem may include more than two branch apical cells in addition to the original apical cell (e.g., Fig. 2E, F). This occurs in nearly half of the individuals with a single apical cell (33% to 61%), and suggests that this type of branch apical cell formation is relatively common in thalli of *Mizutania*.

Not all branch apical cells continue to function as initial cells, and some lose this function by periclinal divisions soon after initiation. In Fig. 2 E and F, the second-youngest branch apical cell has undergone periclinal division, while in Fig. 3C, there is a failed branch apical cell with a periclinal wall in the very small branch thallus with a non-meristematic apex, suggesting that the branch apical cell loses its function through periclinal division. There appears to be no general rule that governs which branch apical cells are lost and which grow. To determine the frequency of failed branch apical cells, 30 of 61 individuals were chosen with both original and branch apical cells, and five of these had failed branch apical cells with periclinal walls, indicating that at least 17% of branch apical cells are lost.

In rare cases (9 of 186 individuals), the original apical cell ceased to function by undergoing periclinal and anticlinal division (Fig. 3D, E). Cessation of main apical meristem activity would lead to a fully differentiated thallus with no apical meristem (Fig. 1C).

Adventitious meristem formation

In very rare cases (3 of 186 individuals), a group of small meristematic cells was found on the apical flank (Fig. 3F, G), but no apical cells could be distinguished in this area. The origin of this meristem could not be traced to any merophytes from the original apical cell (Fig. 3G), indicating that this meristem had no relation to the apical segmentation pattern, and was thus termed an adventitious meristem. In Fig. 3I, an adventitious meristem occupies the summit of a thallus (Fig. 3H, I), but we do not know if it would later form an apical cell that could grow further.

Regenerant bud formation

One marginal cell of the thallus, which was somewhat differentiated, produced apical cells by successive oblique divisions (Fig. 3J). The apical cell behaved as a single apical cell, giving rise to obovate lobes (Fig. 3K). It appears likely in this case that a regenerant bud had detached from the original thallus and had grown into a new individual thallus with a single apical cell.

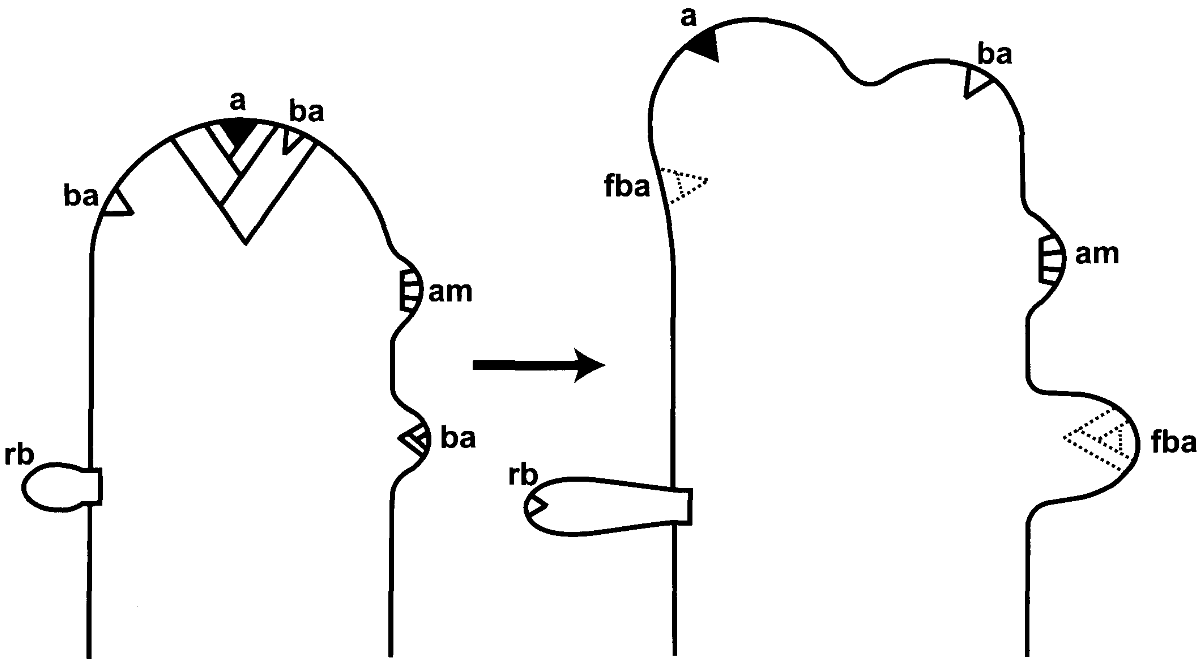

FIG. 4. Diagram of branching modes in thallus of *Mizutania riccardioides*. Dotted lines indicate failed apical cell and meristem. a, apical cell; am, adventitious meristem; ba, branch apical cell; fba, failed branch apical cell; rb, regenerant bud.

Discussion

The resemblance between the thalli of *Mizutania* and fern gametophytes has been stressed based on gross morphological traits (Furuki & Iwatsuki 1989) and meristem behavior (Crandall-Stotler *et al.* 1994). Crandall-Stotler *et al.* (1994) reported that notch apical initials in the shallow concave region of the thallus of *Mizutania* are comparable to plural apical initials (Raghavan 1989) in the notch region of fern gametophytes. However, we could not confirm the existence of these notch apical initials in the thallus of *Mizutania*, even in thalli that were just beginning to fork (e.g., Fig. 3B).

Crandall-Stotler *et al.* (1994) indicated that the loss of notch apical initials contributes to thallus branching. However, we could not confirm this, but instead showed that the branch apical cell was commonly formed in the young merophyte of the original apical cell. This mode of branch apical cell formation is well known in Jungermanniales (Schuster 1984) and other liverworts (Crandall-Stotler 1981, Renzaglia 1982). In this context, thalli of *Mizutania* branch laterally, with a branching mode typical of bryophytes.

The unique characteristics typical of the thalli of *Mizutania* appear after the branch apical cell is established (Fig. 4). Some branch apical cells are lost through periclinal divisions, but there do not seem to be rules as to which branch apical cells grow and for how long the selected branch apical cells continue to function. The arbitrary loss of meristematic activity results in a wide variety of sizes of the branch thalli. The original apical cell itself can fail, and, in addition to branch apical cell formation, some adventitious meristems with no apparent apical cells are formed at low frequency. To our knowledge, this type of adventitious meristem has not been reported in other liverworts.

Morphologically, the thallus of *Mizutania* has been compared to a ribbon-like fern gametophyte. When young, both *Mizutania* and ribbon-

like fern gametophytes have a single apical cell (Masuyama 1975 for *Pleurosoriopsis*). Data on the branching mode of ribbon-like fern gametophytes are fragmentary. Some authors have suggested that, when branched, the single apical cell is probably not retained in ribbon-like fern gametophytes, but instead, the marginal meristem, which lacks an apical cell and consists of equal sized rectangular cells, is formed (Stone 1965 for Hymenophyllaceae, Farrar 1974 for Vittariaceae). The marginal meristem in ferns divides to form two thallus lobes, in association with loss of activity in the central region. A similar marginal meristem resulting thallus branching was not found in *Mizutania*. Hence, the thalli of *Mizutania* are not comparable to ribbon-like fern gametophytes.

In bryophytes, irregularly branched, thalloid gametophytes are rare, occurring only in the protonema phase in the species of *Sphagnum*. In the gametophytes of *Sphagnum*, the apical cell is formed first in the filamentous protonema, and is then replaced by the marginal meristem in the thalloid protonema (Noguchi 1958, Nishida 1970). It appears likely that the marginal meristem is involved in the irregular branching of the *Sphagnum* thalloid protonema. Although the thalli of *Sphagnum* and *Mizutania* are similar in gross morphology, there is a large difference in meristem behavior between the two.

Branched thalloid protonemata also occur in some genera of Jungermanniales (*sensu lato*; Nehira 1966, 1983, Schuster 1991, Gradstein *et al*. 2006). Unfortunately, little attention has been paid to the development of protonema branches since Goebel's (1893) classic work on *Lejeunea metzgeriopsis* (*Metzgeriopsis pusilla*). He presented a figure in which probable branch apical cells are located on both sides of the main apical cell (Fig. 13 in Goebel 1893). Although our examination suggests that arbitrary loss of branch apical cells may have played a key role in the evolution of the highly irregular shape of the thalli of *Mizutania*, the evolutionary pathway remains to be resolved. It is necessary to compare the precise modes of development of thallus branching among the Jungermanniales and related taxa.

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